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CD20-Specific Immunoligands Engaging NKG2D Enhance γδ T Cell-Mediated Lysis of Lymphoma Cells

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Abstract

Human γδ T cells are innate-like T cells which are able to kill a broad range of tumour cells and thus may have potential for cancer immunotherapy. The activating receptor natural killer group 2 member D (NKG2D) plays a key role in regulating immune responses driven by γδ T cells. Here, we explored whether recombinant immunoligands consisting of a CD20 single-chain fragment variable (scFv) linked to a NKG2D ligand, either MHC class I chain-related protein A (MICA) or UL16 binding protein 2 (ULBP2), could be employed to engage γδ T cells for tumour cell killing. The two immunoligands, designated MICA:7D8 and ULBP2:7D8, respectively, enhanced cytotoxicity of ex vivo-expanded γδ T cells against CD20-positive lymphoma cells. Both Vδ1 and Vδ2 γδ T cells were triggered by MICA:7D8 or ULBP2:7D8. Killing of CD20-negative tumour cells was not induced by the immunoligands, indicating their antigen specificity. MICA:7D8 and ULBP2:7D8 acted in a dose-dependent manner and induced cytotoxicity at nanomolar concentrations. Importantly, chronic lymphocytic leukaemia (CLL) cells isolated from patients were sensitized by the two immunoligands for γδ T cell cytotoxicity. In a combination approach, the immunoligands were combined with bromohydrin pyrophosphate (BrHPP), an agonist for Vδ2 γδ T cells, which further enhanced the efficacy in target cell killing. Thus, employing tumour-directed recombinant immunoligands which engage NKG2D may represent an attractive strategy to enhance antitumour cytotoxicity of γδ T cells.

Introduction

Human γδ T cells are innate-like T lymphocytes that recognize stressed cells in the context of infection or malignant transformation [1, 2]. In peripheral blood, γδ T cells represent 1–5% of CD3-positive lymphocytes. While up to 90% of peripheral blood, γδ T cells carry a distinct Vγ9Vδ2 TCR, the majority of intra-epithelial γδ T cells express Vδ1 paired with various Vγ chains [3]. In contrast to αβ T cells, γδ T cells are activated by non-peptide antigens independently of antigen processing and MHC presentation. Whereas ligands for the Vδ1 TCR are not well defined, Vγ9Vδ2 T cells were shown to recognize certain sets of phosphorylated intermediates of the isoprenoid pathway commonly referred to as phosphoantigens (PAg), which are presented by tumour cells in a manner not yet fully understood with butyrophilin subfamily 3 member A1 (CD277) playing an important role [4, 5].

Recent studies indicate that human CD277 molecules, which play key mandatory roles in the PAg-induced activation of Vγ9Vδ2 T cells, do not directly present PAg compounds to T cells. Data from Sebestyen and colleagues support an inside-out signalling mechanism for CD277 whereby intracellular PAg accumulation is translated into surface changes of CD277 by small GTPase RhoB resulting in recognition of the extracellular CD277 domain by Vγ9Vδ2 TCR [6].

Apart from the TCR, tumour cell recognition by γδ T cells involves germline-encoded killer activating receptors including natural killer group 2 member D (NKG2D) [7]. NKG2D, which is also expressed by NK cells and CD8-positive αβ T cells, recognizes multiple stress-inducible self-molecules including MHC class I chain-related protein (MIC) A and B and unique long-16 binding proteins (ULBP) 1–6 [8–10]. The NKG2D ligands are rarely expressed on healthy tissues, but are frequently upregulated

upon cellular stress such as malignant transformation [11]. Consequently, NKG2D ligands function as danger signals enabling recognition and elimination of tumour cells by cytotoxic lymphocytes. Interestingly, some NKG2D ligands (i.e. MICA, ULBP3, ULBP4) were shown to be recognized also by a number of Vδ1 and Vδ2 TCRs, suggesting that these danger signals might trigger γδ T cell activation both *via* TCR as well as NKG2D [12–16]. Whereas it is widely appreciated that ligation of NKG2D by corresponding ligands directly triggers NK cell cytotoxicity and co-stimulates αβ T cells, the relative contribution of NKG2D versus the TCR for γδ T cell cytotoxicity is not precisely clear [10, 11, 17, 18]. The observation that γδ T cell cytotoxicity was induced by NKG2D engagement also in the absence of TCR signalling suggested that like in NK cells, NKG2D may function as a primary activating receptor in γδ T cells [15, 19]. In addition, NKG2D signalling may enable γδ T cells to discriminate malignant from normal cells [20].

Several important functions of γδ T cells in tumour immune surveillance have been demonstrated in murine tumour models [21–23]. Moreover, the numbers of circulating γδ T cells were found to be increased in certain patients with haematological malignancies including chronic lymphocytic leukaemia (CLL), and observed dysfunctional Vγ9Vδ2 T cells were reported to be a negative prognostic factor in CLL – suggesting that these T cells contribute to the control of the disease in humans [24–26]. In clinical trials, the potential of γδ T cell-based cancer immunotherapy was investigated [1]. To this end, γδ T cells were activated *ex vivo* and expanded using PAg such as 2-methyl-3-butenyl-1-pyrophosphate (2M3B1-PP), bromohydrin pyrophosphate (BrHPP) or aminobisphosphonates (n-BP), and then adoptively transferred into the patients. In other approaches, *in vivo* stimulation of γδ T cells by administration of IL-2 or agonistic n-BPs such as pamidronate, zoledronate or PAg phosphostim (IPH1101) were evaluated [27–33]. Although these studies revealed a clinical benefit in some patients, novel strategies further enhancing the antitumoral activities of γδ T cells are warranted.

Increasing the surface density of NKG2D ligands on the surface of malignant cells may provide an innovative strategy to enhance antitumoral activities by NKG2D-positive NK and T cells and may allow boosting γδ T cell cytotoxicity [34]. Previously, in an attempt to attract NK cells, two recombinant immunoligands containing a CD20 single-chain fragment variable (scFv) and a NKG2D ligand (i.e. ULBP2 or MICA) were applied to decorate lymphoma and leukaemia cells exogenously with NKG2D ligands, which resulted in enhanced NK cell-mediated tumour cell killing [35, 36]. However, the abilities of such recombinant immunoligands to trigger other NKG2D-positive, cytotoxic effector cell populations apart from NK cells have

not been investigated in detail. On the basis of the role of NKG2D ligands in the regulation of γδ T cell cytotoxicity, we hypothesized that tumour cell-directed NKG2D ligands could be able to enhance γδ T cell cytotoxicity as well. Thus, we investigated the two recombinant CD20-directed immunoligands for their ability to enhance cytotoxic activities of γδ T cells against B-lineage lymphoma or leukaemia cells.

Materials and methods

Cell culture. Ramos, Raji, Daudi and CEM cells (DSMZ, the German Resource Centre for Biological Material; Braunschweig, Germany) were cultured in RPMI 1640 GlutaMax-I medium (Invitrogen, Life Technologies, Karlsruhe, Germany) supplemented with 10% foetal calf serum (FCS; Invitrogen, Life Technologies), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Life Technologies). INA-6 myeloma cells were cultured as described [37]. Lenti-X 293T cells (Clontech, Saint-Germain-en-Laye, France) were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Life Technologies) containing 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin, and CHO-K1 cells (DSMZ) in chemically defined CHO medium (Invitrogen, Life Technologies) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin and HT supplement (Invitrogen, Life Technologies).

Preparation of γδ T cells. The Department of Transfusion Medicine in Kiel, Germany, provided leukocyte concentrates from healthy adult blood donors, who gave their informed consent. Additionally, heparinized blood was provided from healthy donors. The research was approved by the relevant institutional review board (code number: D405/10 und 404/14). Briefly, PBMC were isolated by Ficoll–Hypaque gradient density centrifugation. PBMC were cultured in RPMI 1640 containing 2 mmol/l L-glutamine, 25 mmol/l HEPES, antibiotics and 10% FCS. To obtain short-term activated Vγ9Vδ2 γδ T cells, PBMC were stimulated with 300 nmol/l of PAg BrHPP (kindly provided by Innate Pharma, Marseille, France) and 50 U/ml rIL-2 (Novartis, Basel, Suisse). IL-2 was added every 2 days over a period of 2–3 weeks. γδ T cells with a lower purity were subjected to magnetic separation to deplete remaining non-γδ T cells. Short-term activated Vδ1 γδ T cells were generated by staining PBMC with anti-TCRVδ1 mAb (Immunotech/Beckman Coulter, Krefeld, Germany) followed by positive selection with Dynabeads® (Dynal, Thermo Fisher Scientific). Magnetically isolated Vδ1 γδ T cells as well as several short-term activated Vδ2 γδ T cells (Table S1) were re-stimulated with feeder cells, which included 40 grey irradiated PBMC (1:2 ratio) and 60 grey irradiated EBV-transformed B cell lines (1:10 ratio) as well as 0.5 μg/ml PHA and 50 U/ml rIL-2. The purity of the cells was determined 2–3 weeks after initial stimulation or

re-stimulation by flow cytometry. Most γδ T cell preparations had a purity >97% (Table S1).

Recombinant immunoligands. The recombinant immunoligands MICA:7D8 and ULBP2:7D8, which each contain the human CD20 scFv 7D8 and either the extracellular part of MICA or ULBP2, were constructed as described previously [35]. Expression vectors for control proteins were generated by replacing the sequences encoding the scFv 7D8 [38] by sequences coding for either the CD7 scFv TH69, or an scFv specific for HM1.24 in the expression vectors pSecTag2/MICA:7D8-myc-his and pSecTag2/ULBP2:7D8-myc-his [35, 39, 40]. Correct sequences were confirmed by Sanger sequencing. The proteins were expressed and purified as described [35]. SDS-PAGE and Western transfer experiments were performed according to standard procedures using mouse antipenta-His (Qiagen, Hilden; Germany) and secondary horseradish peroxidase-conjugated goat anti-mouse IgG antibodies for detection (Dianova, Hamburg, Germany). Immunoligands were deglycosylated under denaturing reaction conditions using Protein Deglycosylation Mix (New England BioLabs, Frankfurt, Germany) containing the enzymes O-glycosidase, PNGase F, neuraminidase, β1-4 galactosidase and β-N-acetylglucosaminidase according to the manufacturer's instructions. For Western blotting, 1.5 μg of proteins were loaded onto a gel.

Homology models. Separate homology models were calculated for the scFv 7D8 and the extracellular domains of MICA and ULBP2 using YASARA Structure software (YASARA Biosciences, Graz, Austria) after removal of sequences for the corresponding secretion leaders and C-terminal tags. The model structure for the whole fusion proteins were then obtained by fusing the best-fitting models for the single subunits after introducing the linker sequences. Ribbon drawings were generated using Discovery Studio 2.0 Visualizer software (Accelrys, San Diego, CA, USA).

Flow cytometric analysis. Flow cytometry was performed on flow cytometers FC 500 or Navios (Beckmann Coulter, Brea, CA, USA) as described [36]. The hexa-histidine-tagged recombinant immunoligands were detected with a secondary Alexa Fluor 488-conjugated antipenta-His antibody (Qiagen). Alternatively, cell-bound immunoligands and the endogenously expressed MICA/B and ULBP2 were analysed with phycoerythrin (PE)-labelled antibodies (R&D Systems, Wiesbaden, Germany). Unless otherwise indicated, MICA:7D8 and ULBP2:7D8 were used at 15 μg/ml. Apparent CD20 binding affinities were derived from dose−response curves. CD20 expression on primary tumour cells was verified with a FITC-conjugated antibody obtained from BD Biosciences (Heidelberg, Germany). γδ T cell lines were characterized with the following mAb: CD3 (clone SK7; BD Biosciences), anti-TCRγδ (clone 11F2; BD Biosciences), anti-TCRVδ1 (clone TS8.2; Thermo Fisher Scientific, Karlsruhe, Germany), anti-TCRVδ2 (clone Immu389; Beckman Coulter) and anti-NKG2D (clone 149810; R&D Systems). Appropriate isotype antibodies were used as controls.

Cytotoxicity assay. Cytotoxic properties of MICA:7D8 and ULBP2:7D8 were investigated in standard ^{51}Cr-release experiments performed in 96-well microtiter plates as described [36]. Briefly, 1×10^6 target cells were labelled with 100 μCi of ^{51}Cr for 2 h. Cells were washed three times and the cell number was adjusted to 1×10^5 cells/ml. Medium (100 μl) and immunoligands at the indicated concentrations were added to round bottom microtiter plates before γδ T cells (50 μl) and target cells (50 μl) were applied, resulting in a final volume of 200 μl. After 3 h at 37 °C, assays were stopped by centrifugation. Twenty-five microlitre of supernatant were mixed with 125 μl scintillation solution (OptiPhase Supermix; Perkin Elmer, Waltham, USA), incubated with agitation for 15 min and analysed with a beta counter. ^{51}Cr release from triplicates was measured in counts per minute (cpm). Percentage of γδ T cell cytotoxicity was calculated using the formula: % specific lysis = (experimental cpm − basal cpm) / (maximal cpm − basal cpm) × 100. Maximal ^{51}Cr release was determined by adding Triton X-100 (1% final concentration) to target cells, and basal release was measured in the absence of sensitizing antibody constructs and γδ T cells. Unless otherwise indicated, experiments were performed in the presence of 50 U/ml IL-2 employing γδ T cells as effector cells at an effector-to-target (E:T) cell ratio of 100:1. γδ T cell lines were used 10–12 days after initial stimulation. In selected experiments, BrHPP (Innate Pharma) was added to a final concentration of 300 nM.

Data processing and statistical analyses. Graphical and statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P-values were calculated using the student's t-test or repeated measures ANOVA and Bonferroni post-test when appropriate. The null hypothesis was rejected for $P < 0.05$.

Results

Characterization of CD20−directed immunoligands MICA:7D8 and ULBP2:7D8

To evaluate NKG2D as a trigger molecule on γδ T cells, two recombinant immunoligands consisting of the human CD20 scFv 7D8 fused to a NKG2D-specific ligand, either MICA or ULBP2 (Fig. 1A, Figure S1A), were employed, which had been recently generated to recruit NK cells against lymphoma or leukaemia cells [35]. Biochemical characterization indicated that MICA:7D8 and ULBP2:7D8 were glycosylated proteins of approx. 90–110 and 55–65 kDa, respectively (Fig. 1B, C), which behaved as monomers in solution (Figure S1B). Both immunoligands specifically bound to CD20-positive
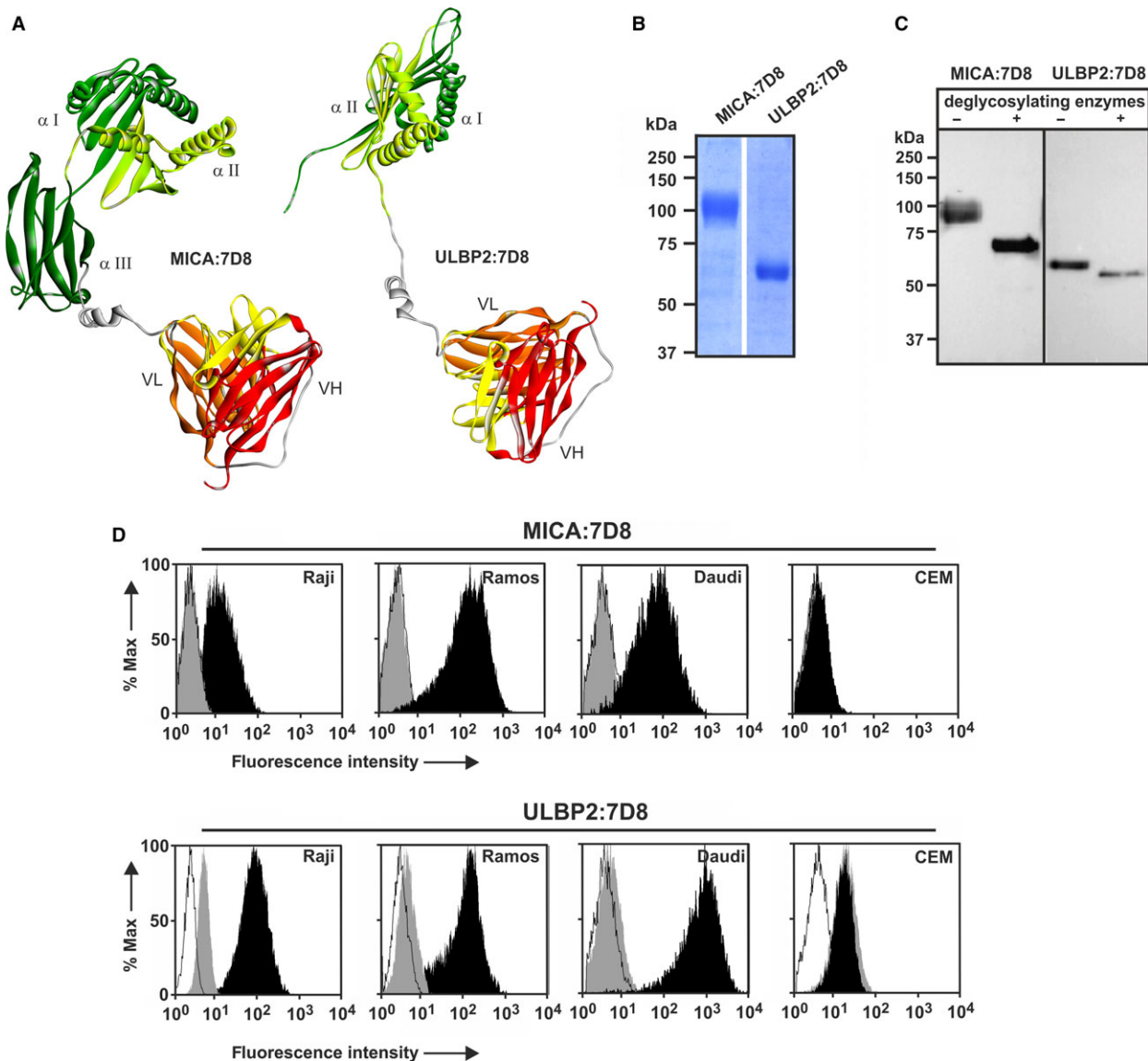
Figure 1 Characteristics of MICA:7D8 and ULBP2:7D8. (A) Ribbon drawing according to homology models of ULBP2:7D8 and MICA:7D8. The ligands are coloured in green. MICA displays α I–III domains similar to those comprised in the heavy chains of MHC class I molecules, while only α I and α II domains are contained in ULBP2. V_H and V_L regions of scFv 7D8 are depicted in red and orange, respectively. Yellow-stained domains indicate the complementarity determining regions. (B) Integrity of purified MICA:7D8 (lane 1) and ULBP2:7D8 (lane 2) expressed in CHO K1 cells was analysed by SDS gel electrophoresis under reducing conditions and subsequent staining with coomassie brilliant blue. (C) Purified MICA:7D8 and ULBP2:7D8 were either deglycosylated under denaturing conditions (+) by applying a mixture of enzymes (O-glycosidase, PNGase F, neuraminidase, β1-4 galactosidase and β-N-acetylglucosaminidase) or left untreated (−), and subsequently analysed by Western blot transfer experiments using an antipenta histidine antibody. One representative experiment of two performed is shown. (D) Coating of lymphoma cell lines with MICA:7D8 or ULBP2:7D8 increases the surface density of the corresponding NKG2D-specific ligands. Cells were first coated with MICA:7D8 (upper panel, black peaks) and ULBP2:7D8 (lower panel, black peaks) or left untreated (grey peaks), and subsequently stained with PE-coupled antibodies specific for MICA/B (upper panel) or ULBP2 (lower panel) and analysed by flow cytometry. Cellular debris and dead cells were excluded by setting appropriate scatter gates. Endogenous expression of respective ligands is reflected by the signals obtained with antibodies recognizing the NKG2D ligands in samples with untreated cells (grey peaks). As a control, untreated cells were incubated with a PE-conjugated control antibody (white peaks). CD20-negative CEM cells served as a control. Note that CEM cells express considerable amounts of endogenous surface ULBP2. One experiment of two performed is shown.

lymphoma cells, and binding was partially blocked by a recombinant Fab fragment derived from the parental antibody 7D8 (Figure S1C). Analysis of apparent affinities to CD20 indicated that the scFv 7D8-moiety contained in both fusion proteins had similar affinities, regardless whether it was fusesd to MICA or ULBP2 (Figure S1D). Calculated K_D-values were $1.6 \pm 0.1 \times 10^{-7}$ M and $1.9 \pm 0.1 \times 10^{-7}$ M for MICA:7D8 and ULBP2:7D8,

respectively. Finally, we determined the increase in surface-exposed MICA and ULBP2 that was achieved by coating the cells with the immunoligands (Fig. 1D). To this end, the lymphoma cell lines Raji, Ramos and Daudi as well as the CD20-negative T cell leukaemia cell line CEM were either pre-incubated with the immunoligands, or left untreated, and subsequently stained with fluorescence-conjugated antibodies against MICA/B or ULBP2. Endogenous cell surface expression of MICA/B and ULBP2 varied between the cell lines, but was moderate or not detectable. Significantly, pre-incubation with MICA:7D8 and ULBP2:7D8 increased the amounts of MICA and ULBP2 on the surface of the CD20-positive lymphoma cell lines by 1–2 orders of magnitude (Fig. 1D).

ULBP2:7D8 and MICA:7D8 enhance γδ T cell–mediated lysis of lymphoma or leukaemia cells

To analyse the ability of MICA:7D8 and ULBP2:7D8 to enhance γδ T cell cytotoxicity, a panel of γδ T cell lines were established from PBMC of healthy donors (Table S1). The resulting short-term activated or re-stimulated γδ T cell lines, which included both Vδ1 or Vδ2 cell lines also strongly expressing NKG2D (Figure S2), were then analysed as effector cells for MICA:7D8 and ULBP2:7D8. As a result, both ULBP2:7D8 and MICA:7D8 enhanced lysis of Raji lymphoma cells significantly in the presence of Vδ2 γδ T cells (Fig. 2A). In contrast, no cytotoxic effects were induced by the immunoligands in the absence of effector cells, illustrating that cytotoxicity mediated by both MICA:7D8 and ULBP2:7D8 was effector cell dependent. Moreover, MICA:7D8 and ULBP2:7D8 did not mediate killing of CD20-negative cells, as demonstrated using the CD20-negative plasmacytoma cell line INA-6 (Fig. 2B). Therefore, both MICA:7D8 and ULBP2:7D8 triggered γδ T cells in a target antigen restricted manner.

To further study the enhancing effect of MICA:7D8 and ULBP2:7D8, cytotoxicity experiments were performed using Raji cells as target cells and Vδ2 γδ T cells at varying E:T cell ratios (Fig. 2C). Lysis induced by MICA:7D8 and ULBP2:7D8 was enhanced with increasing E:T cell ratios. Similar results were obtained when Vδ1 γδ T cells were employed although the extent of lysis was lower (Fig. 2D). Furthermore, dose-dependent induction of cytotoxicity was demonstrated for the immunoligands employing different Vδ2 γδ T cell lines and Raji or Ramos lymphoma target cells (Fig. 2E). Both MICA:7D8 and ULBP2:7D8 were active at nanomolar concentrations. In experiments with different γδ T cell lines, MICA:7D8 had mean half-maximum effective concentrations (EC50) of 0.04 μg/ml (~0.6 nm) and 0.22 μg/ml (~3.3 nm) for Raji and Ramos cells, respectively. ULBP2:7D8 had an EC50 of 0.09 μg/ml (~1.8 nm) for Raji and an EC50 of 0.3 μg/ml (~6.0 nm) for Ramos cells.

Moreover, cytotoxic effects induced by the immunoligands were demonstrated using various Vδ2 or Vδ1 γδ T cell lines and different Burkitt lymphoma cell lines including Raji, Ramos and Daudi (Fig. 3A, B, C, respectively). Whereas none of the cell lines expressed detectable levels of endogenous MICA, ULBP2 was expressed in significant amounts by Raji cells and poorly displayed by Ramos and Daudi (Fig. 1D). Of note, different γδ T cell lines differed in their responses to stimulation with the immunoligands suggesting that additional parameters may play an important role, which have not yet been determined.

Furthermore, the properties of MICA:7D8 and ULBP2:7D8 to trigger cytotoxicity of γδ T cells against freshly isolated CLL cells were analysed (Fig. 4). Using different Vδ2 γδ T cell lines, both MICA:7D8 and ULBP2:7D8 induced efficient lysis of CLL cells from different patients, whereas similarly constructed control molecules were ineffective (Fig. 4A, B). Lysis was induced in a dose-dependent manner and the immunoligands triggered cytotoxicity at nanomolar concentrations, similar to results obtained with lymphoma cell lines (Fig. 4C). Mean EC50 values were approx. 0.6 μg/ml (~9 nm) and 0.2 μg/ml (~4 nm) for MICA:7D8 and ULBP2:7D8, respectively.

To provide an additional stimulus for the effector cells, the immunoligands were analysed in combination

Figure 2 MICA:7D8 and ULBP2:7D8 sensitize lymphoma cells for γδ T cell-mediated lysis. (A) MICA:7D8 and ULBP2:7D8 were analysed for the ability to trigger γδ T cells to kill Raji cells in ⁵¹Cr release experiments. No cytotoxicity was observed in the absence of γδ T cells. The immunoligands were applied at a concentration of 10 μg/ml. E:T cell ratio was 100:1. Data points represent mean values ± SEM from three independent experiments using different Vδ2 T cell lines (*P < 0.05; BR, basal release). (B) ULBP2:7D8 and MICA:7D8 (10 μg/ml) do not trigger γδ T cell-mediated killing of CD20-negative multiple myeloma INA-6 cells (E:T cell ratio: 100:1). The similarly designed immunoligand ULBP2:HM1.24, which targets the CD317 (HM1.24) antigen being expressed by INA-6, was used as a positive control. Data are presented as mean ± SEM using different Vδ2 T cell lines (*P < 0.05; BR, basal release; n = 3). (C) Killing of Raji cells by MICA:7D8 (left) and ULBP2:7D8 (right) at varying E:T cell ratios employing Vδ2 γδ T cells. Cytotoxicity was analysed in ⁵¹Cr release experiments. The immunoligands were applied at a concentration of 10 μg/ml. Data points represent mean values from six independent experiments (*P < 0.05 relative to the control groups). (D) Killing of Raji cells by MICA:7D8 (left) and ULBP2:7D8 (right) at varying E:T cell ratios employing Vδ1 γδ T cells. Data points represent mean values from four independent experiments (*P < 0.05 compared with control groups). (E) Dose-dependent killing of Raji (left panel) and Ramos cells (right panel) by MICA:7D8 and ULBP2:7D8. Vδ2-expressing γδ T cell lines were employed as effector cells at an E:T cell ratio of 100:1. Similarly designed immunoligands containing a scFv against an antigen not expressed by B-lymphoid tumour cells (contr.-scFv) were used as control. Data points represent mean values ± SEM from at least three different experiments with different γδ T cell lines (*P < 0.05 compared with control immunoligand).
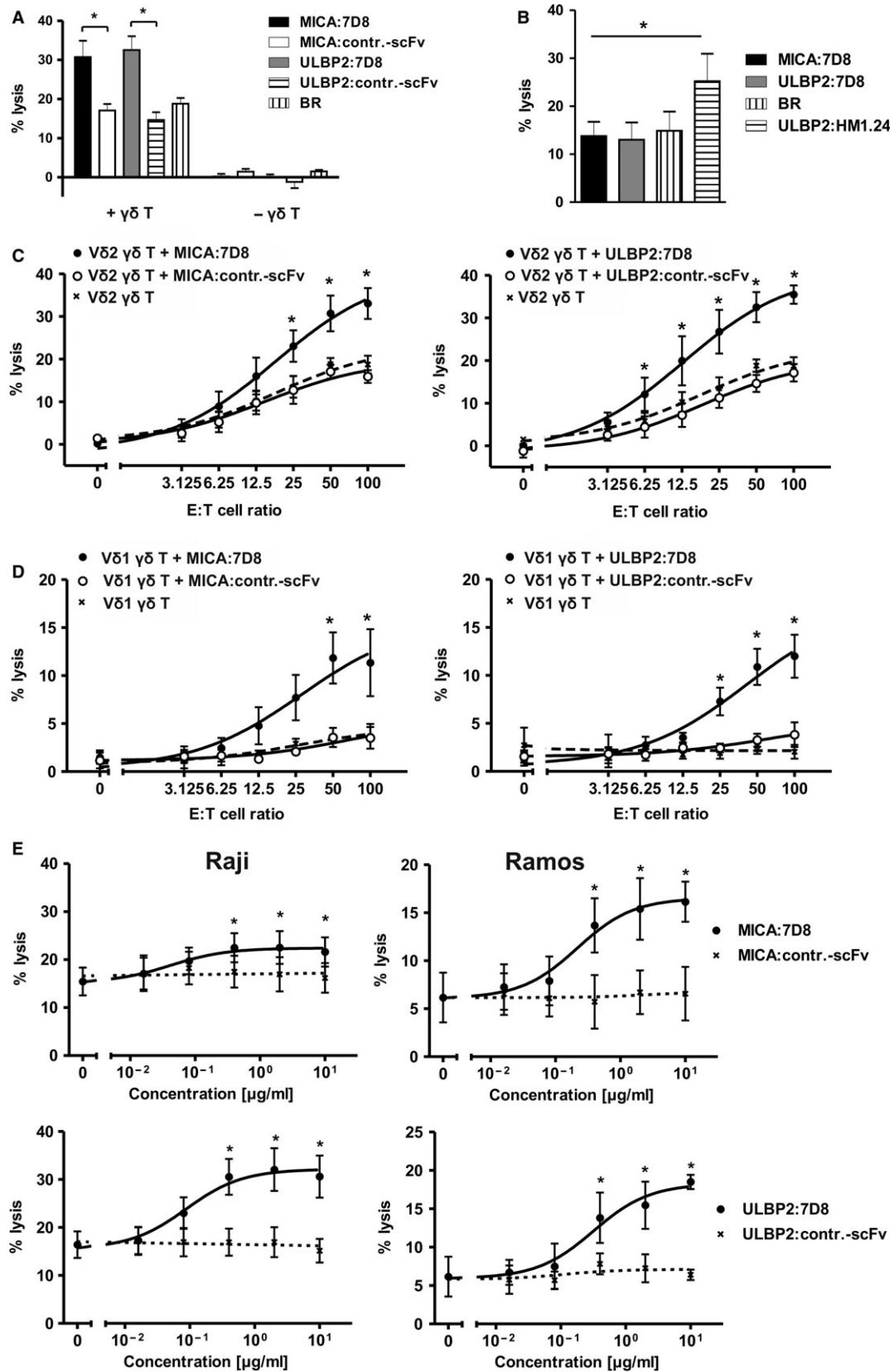
with BrHPP, an agonist for the Vδ2Vγ9 γδ TCR, which in combination with IL-2 was shown to induce a potent expansion of γδ T lymphocytes in patients with solid tumours [33]. BrHPP efficiently promoted cytotoxicity of different Vδ2Vγ9 γδ T cell lines against Raji cells (Fig. 5A). Interestingly, through the combination of BrHPP with ULBP2:7D8 or MICA:7D8, lysis of target cells was further enhanced (Fig. 5A, B). Finally, the combination of the immunoligands with BrHPP was also tested with patient-derived CLL cells (Fig. 5C, D). BrHPP enhanced killing of CLL cells by Vδ2 γδ T cells (Fig. 5C), but killing efficacy (~10% lysis in the mean) remained low in contrast to experiments performed with Raji cells (~40% Fig. 5A), despite strong surface expression of CD277 (Figure S3), which plays an important role in PAg presentation [4, 5]. Impressively, both MICA:7D8 and ULBP2:7D8 produced significant additional cytotoxic effects (Fig. 5A, B, C). Thus, the magnitude of Vδ2 γδ T cell-mediated lysis of CLL cells was significantly enhanced by combining the immunoligands with BrHPP, relative to treatment with the single agents. Again, both MICA:7D8 and ULBP2:7D8 induced cytotoxicity in a dose-dependent manner (Fig. 5D). In summary, the two immunoligands MICA:7D8 and ULBP2:7D8 enhanced the cytotoxic activities of ex vivo-expanded Vδ1 and Vδ2 γδ T cells resulting in effective lysis of CD20-positive lymphoma cell lines and patient-derived CLL cells.

Discussion

In this study, recombinant immunoligands engaging NKG2D were evaluated for their potential to enhance γδ T cell-mediated cytotoxicity. The human CD20-directed fusion proteins MICA:7D8 and ULBP2:7D8 were able to trigger both Vδ1 and Vδ2 γδ T cells and significantly enhanced their cytotoxicity against lymphoma and leukaemia cells. These findings demonstrate that biologicals containing NKG2D-specific ligands may represent an attractive class of molecules to enhance antitumour responses by γδ T cells.

Therapeutic strategies harnessing the antitumoral activities of NK and γδ T cells might be promising approaches to treat solid tumours and haematologic malignancies [1, 2, 34]. Recombinant immunoligands engaging stimulatory surface receptors such as NKG2D, NKp30 or NKp80 and targeting tumour-associated antigens have been shown to stimulate NK cell cytotoxicity against malignant cells [35, 36, 41–43]. Previously, we have demonstrated that MICA:7D8 and ULBP2:7D8, which are devoid of the antibody's Fc domain and thus are unable to trigger complement-dependent cytotoxicity or to engage Fc receptors, triggered NK cells to kill lymphoma or leukaemia by NKG2D engagement [35]. Moreover, the fusion proteins synergistically enhanced antibody-dependent cell-mediated cytotoxicity, an important effector

function of therapeutic antibodies [35, 44]. In this study, we demonstrate that such an approach may be utilized to trigger T cells, because MICA:7D8 as well as ULBP2:7D8 induced lysis of lymphoma or leukaemia cells by ex vivo-activated and expanded γδ T cells. Thus, apart from NK cells, γδ T cells may represent another potential effector cell population triggered by MICA:7D8 and ULBP2:7D8. However, the extent of tumour cell lysis varied between different γδ T cell lines, and it should be noted that with some lines the immunoligands produced only minor effects, which may reflect donor variability or differences in the γδ T cell activation status acquired during the expansion. Noteworthy, the immunoligands enhanced cytotoxicity by γδ T cells even in the presence of BrHPP, which provided an additional activating stimulus to the effector cells, suggesting collaboration between the immunoligands and the phosphorylated antigen. The additional cytotoxic effects were especially pronounced in experiments with CLL cells from patients. Our results indicate that immunoligands selectively targeting γδ T cells to tumour cell-expressed antigens may provide a tool to further enhance cytotoxic activity of γδ T cells where BrHPP failed due to anergy, exhaustion or depletion of γδ T cells [26, 29, 45, 46].

γδ T cells were shown to exert antitumour activities by directly killing malignant cells and by trans-activating cells of both the adaptive and the innate immune system [47, 48]. In particular, γδ T cells were shown to enhance NK cell cytotoxicity either by releasing soluble factors or through cell–cell contact [49, 50]. Considering that NK cells activated by γδ T cells displayed further increased NKG2D expression levels and that the observed augmented lytic NK cell activity was found to be at least in part NKG2D dependent, we anticipate that recruitment of both NK and γδ T cells by NKG2D-directed antibody derivatives may induce positive feedback loops [49].

Similar to results obtained with NK cells [35], MICA:7D8 and ULBP2:7D8 were active in the nanomolar concentration range when γδ T cells were employed as effector cells. There was a trend towards higher activity of ULBP2:7D8 compared to MICA:7D8, although the differences between the two immunoligands with γδ T cell effector cells were not as clear as with NK cells [35]. Notably, in the case of γδ T cells, also a differential recognition of the two NKG2D ligands by different TCR has to be considered. While the Vδ1 γδ TCR was reported to recognize MICA, an interaction with ULBP2 has not been observed [12, 16]. In contrast, Vδ2-expressing γδ TCRs usually bind neither ULBP2 nor MICA.

Apart from NK cells and γδ T cells, NKG2D is also expressed by conventional CD8-positive αβ T cells and some CD4-positive αβ T cells. However, NKG2D is believed to have coactivating rather than stimulatory functions on αβ T cells. Whether αβ T cells are also triggered by NKG2D-specific immunoligands has not
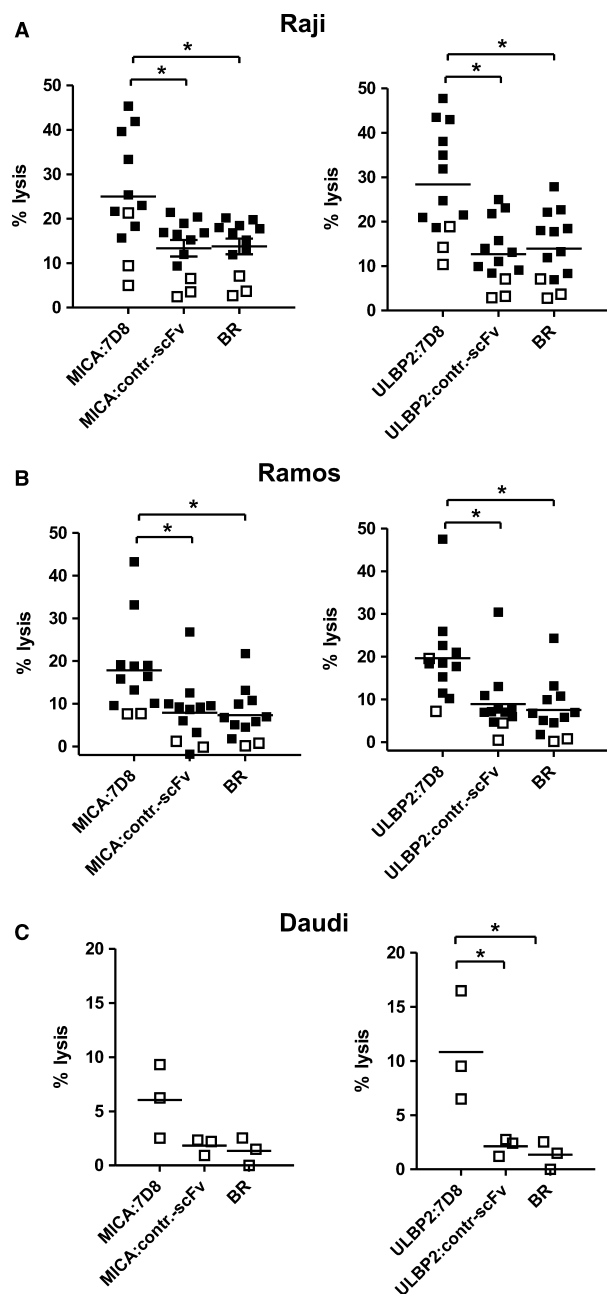
Figure 3 Efficacy of MICA:7D8 and ULBP2:7D8 with different Vδ1 or Vδ2 γδ T cell lines. The cytotoxic activity of the immunoligands was analysed with different Vδ1 (open squares) or Vδ2 γδ T cell lines (filled black squares) employing Raji (A), Ramos (B) and Daudi lymphoma cells (C). Data points represent mean values from triplicate determinations. Similarly, designed immunoligands containing a scFv against an antigen not expressed by B-lymphoid tumour cells (contr.-scFv) were used as control. The immunoligands were employed at a concentration of 10 μg/ml (BR, basal release; *P < 0.05).

Figure 4 MICA:7D8 and ULBP2:7D8 trigger γδ T cell-mediated killing of freshly isolated tumour cells. (A) The cytotoxic activity of the immunoligands against freshly isolated CLL cells was analysed employing Vδ2 γδ T cell lines in a ^{51}Cr release assay. The immunoligands were employed at a concentration of 10 μg/ml. Similarly, constructed immunoligands directed against an antigen not expressed by B-lymphoid tumour cells (contr.-scFv) were used as control. Data points represent mean values from triplicate determinations, error bars indicate SEM. Note that different Vδ2 γδ T cell lines were used in experiments with different target cells. CLL cell samples from four different patients were employed. The E:T cell ratio was 100:1 (BR, basal release; p, patient). (B) For statistical analysis, CLL samples were analysed as a group (*P < 0.05; n = 4). (C) MICA:7D8 and ULBP2:7D8 triggered γδ T cell-mediated killing of CLL cells (CLL p#1) in a dose-dependent manner at nanomolar concentrations. Data points represent mean values from three different experiments using different Vδ2 γδ T cell lines. Statistical significant differences in the extent of lysis achieved by either MICA:7D8 and ULBP2:7D8 or the corresponding control constructs are indicated (*P < 0.05).

been addressed in detail yet. Activation of polyclonal T cells has not been observed after exposure to B-lymphoid tumour cells coated with the immunoligands MICA:7D8 or ULBP2:7D8 [35]. However, it has not been investigated whether MICA:7D8 and ULBP2:7D8 may selectively
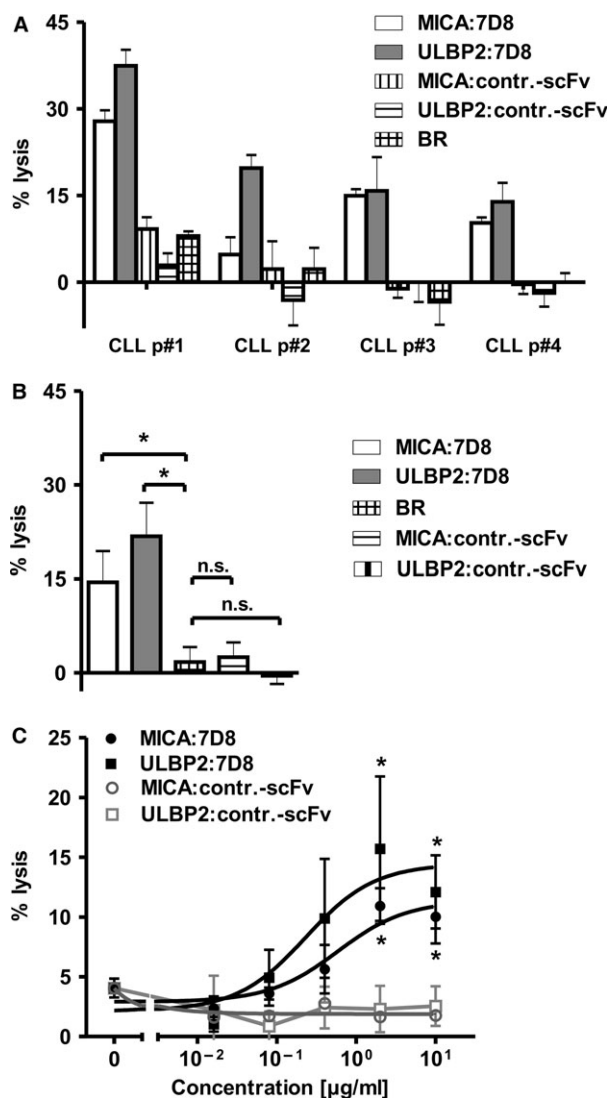
activate certain distinct αβ T cell subsets such as effector memory T cells, or if the induction of cytotoxicity by polyclonal αβ T cells via NKG2D signalling might occur
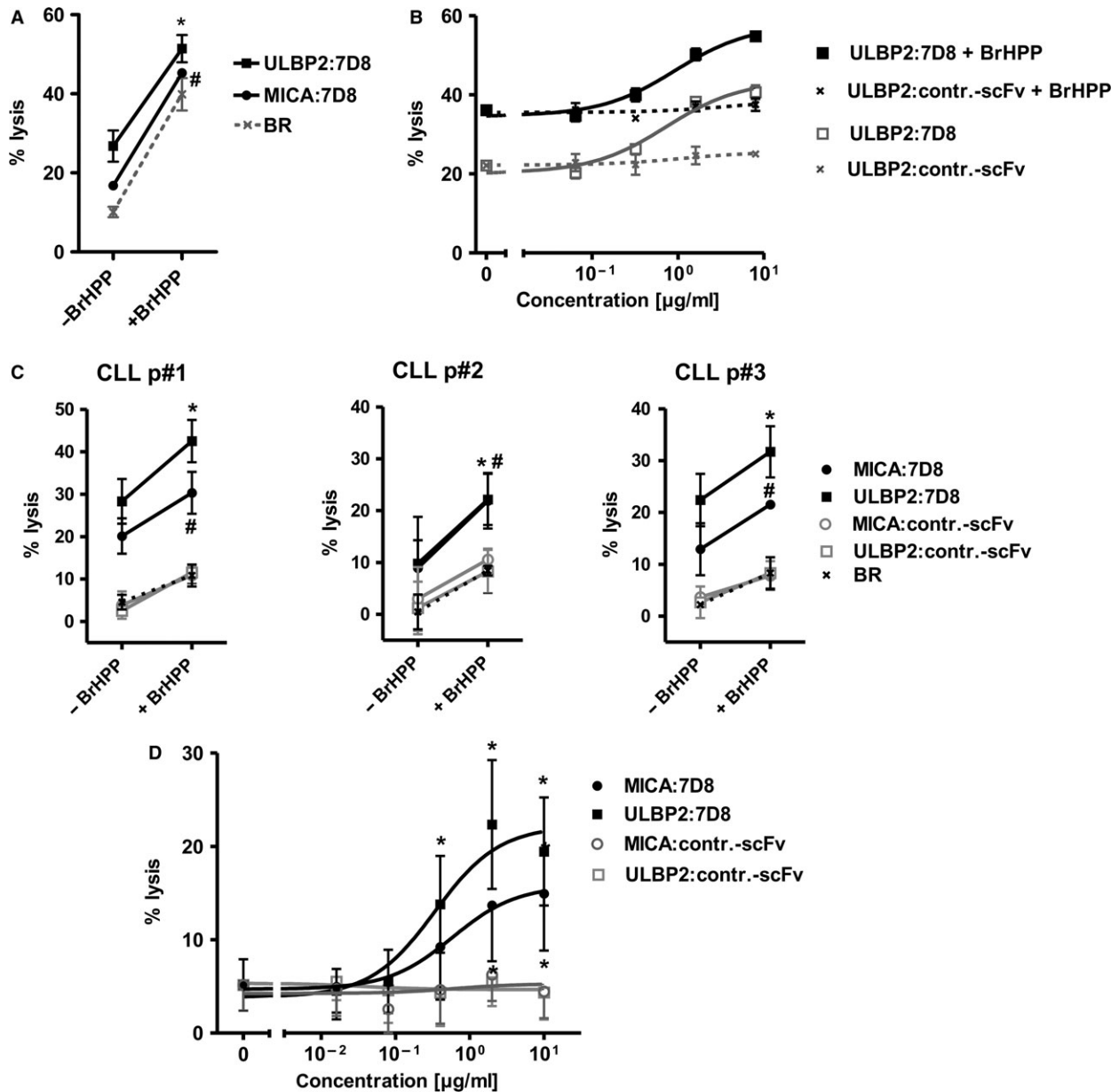
Figure 5 Enhanced Vδ2 γδ T cell-mediated cytotoxicity by combining the immunoligands with BrHPP. (A) Killing of Raji cells by MICA and ULBP2:7D8 was analysed either in the presence or in the absence of BrHPP. Data points indicate mean values ± SEM of four independent experiments. BrHPP significantly enhanced the magnitude of target cell lysis induced by ULBP2:7D8 (*$P < 0.05$) or MICA:7D8 (#$P < 0.05$). The immunoligands were used at a concentration of 10 µg/ml, and BrHPP at 300 nM. (B) Dose-dependent lysis of Raji cells by ULBP2:7D8 either in the presence or in the absence of BrHPP. Data points represent mean values ± SEM from triplicate determinations. (C) Extent of Vδ2 γδ T cell-mediated lysis of CLL cells induced by the immunoligands either in the presence or in the absence of BrHPP. Data points indicate mean values ± SEM of three independent experiments. BrHPP significantly enhanced the magnitude of target cell lysis induced by ULBP2:7D8 (*$P < 0.05$) or MICA:7D8 (#$P < 0.05$). (D) Dose-dependent induction of Vδ2 γδ T cell cytotoxicity against CLL cells (CLL p#1) by MICA:7D8 and ULBP2:7D8 in the presence of BrHPP (concentration: 300 nM). The E:T cell ratio was 100:1. Data points represent mean values ± SEM of three independent experiments (*$P < 0.05$).

under certain circumstances. For instance, upon stimulation with IL-15 CD8-positive αβ T cells have been demonstrated to kill target cells in a TCR-independent but NKG2D-dependent manner [51].

In summary, both MICA:7D8 and ULBP2:7D8 enhanced cytotoxicity by various γδ T cell lines indicating that in addition to NK cells also γδ T cells may constitute a relevant effector cell population for tumour-directed, NKG2D-specific immunoligands. Thus, employing immunoligands such as ULBP2:7D8 or MICA:7D for NKG2D engagement may represent an innovative immunotherapeutic strategy, which may deserve further evaluation.

Acknowledgment

Conflict of interest

PWHIP and JGJvdW are employees of Genmab, a biotechnology company which develops therapeutic monoclonal antibodies, and own Genmab warrants and/or stock. They are named as inventors on several Genmab-owned CD20 antibody patents which have been licensed to GlaxoSmithKline. The authors have no additional conflict of interests.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Characterization of γδ T cell lines.

Figure S1 Generation and CD20 binding properties of MICA:7D8 and ULBP2:7D8.

Figure S2 NKG2D expression by Vδ1 and Vδ2 γδ T cell lines.

Figure S3 CD277 surface expression.